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(54) Title: PLASTID TRANSFORMATION IN ARABIDOPSIS THALIANA (57) Abstract <p>This invention provides methods and compositions for obtaining transplastomic <i>Arabidopsis</i> and <i>Brassica</i> plants. Specifically, the method provides culturing protocols and compositions that facilitate the regeneration of transformed plants following delivery of exogenous, beneficial DNA molecules.</p>		

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PLASTID TRANSFORMATION IN ARABIDOPSIS THALIANA

Pursuant to 35 U.S.C. Section 202(c), it is acknowledged that the United States government has certain rights in the invention describe herein, which was made in part with funds from the National Science Foundation Grant Number, MCB 93-05037.

FIELD OF THE INVENTION

The present invention relates to the field of transgenic plants. Specifically, the invention provides compositions and methods for the transformation of plastids in plants from the *Cruciferae* family.

BACKGROUND OF THE INVENTION

Several publications are parenthetically referenced in this application in order to more fully describe the state of the art to which this invention pertains. Full citations for these references are found at the end of the specification. The disclosure of each of these publications is incorporated by reference in the present specification as though set forth herein in full.

The plastid genome of higher plants is a circular double-stranded DNA molecule of 120-160 kb which may be present in 1,900-50,000 copies per leaf cell (Palmer, 1991; Bendich, 1987). Stable transformation of the tobacco plastid genome (plastome) has been achieved through the following steps: (i) introduction of transforming DNA, encoding antibiotic resistance, by the biolistic process (Svab et al. 1990a; Svab and Maliga 1993) or PEG treatment (Golds et al. 1993; O'Neill et al., 1993), (ii) integration of the transforming DNA by two homologous recombination events and (iii) selective elimination of the wild-type genome copies during repeated cell divisions on a selective medium. Spectinomycin resistance has been used as a selective marker encoded either in mutant plastid 16S ribosomal RNA genes (Svab et al. 1990a; Staub and Maliga 1992; Golds et

al. 1993), or conferred by the expression of an engineered bacterial *aadA* gene (Svab and Maliga 1993). Vectors which utilize *aadA* as a selectable marker gene and target the insertion of chimeric genes into the repeated region of tobacco plastid genome are available (Zoubenko et al., 1994). Selection of plastid transformants by kanamycin resistance, based on the expression of neomycin phosphotransferase (*kan* gene), is more difficult but also feasible (Carrer et al., 1993; Carrer and Maliga, 1995).

To date, stable plastid transformation in higher plants has been reported in tobacco only (reviewed in Maliga, 1993; Maliga et al., 1993). Transplastomic plants from other agriculturally and pharmaceutically important species are highly desirable. Expression of foreign genes of interest in the plastids of higher plants in the family *Cruciferae* provides several advantages over nuclear expression of foreign genes. These are 1) expression of exogenous DNA sequences in plastids eliminates the possibility of pollen transmission of transforming DNA; 2) high levels of protein expression are attainable; 3) the simultaneous expression of multiple genes as a polycistronic unit is feasible and 4) positional effects and gene silencing which may result following nuclear transformation are also eliminated.

SUMMARY OF THE INVENTION

The present invention provides improved methods for the generation of stably transformed, transplastomic plants. In one embodiment of the invention, cotyledon cells are cultured in high auxin liquid medium for a sufficient time period to stimulate uniform cell division. Initial culture is at a high density (50-200 cotyledons/ 20 ml). The cotyledons are then transferred to agar-solidified medium for delivery of exogenous, transforming DNA. Following delivery of transforming

DNA, the cotyledons are transferred at a lower density (25-30/50 ml) to a medium containing high cytokinin levels and the selection agent to facilitate selection of transformants and plant regeneration. Presence of the
5 exogenous DNA in the plastid genome is then confirmed by Southern blot analysis or PCR.

The transforming DNA molecules of the invention have several distinct features. These are 1) targeting segments flanking the foreign gene of interest consisting
10 of plastid DNA sequences from the plant to be transformed, thereby facilitating homologous recombination of the transforming DNA into a pre-determined region of the plastid genome; 2) a selectable marker gene disposed within the targeting segment,
15 conferring resistance to a selection agent; 3) 5' and 3' regulatory sequences derived from plastid DNA operably linked to sequences encoding a foreign gene of interest thereby enhancing expression of the transforming DNA and stability of encoded mRNA; and 4) at least one cloning
20 site adjacent to the selectable marker gene for insertion of the foreign gene of interest which by itself is not selectable. Since the selectable marker gene and the foreign gene of interest form a heterologous block of contiguous sequence, integration of both genes into the
25 plasid genome is effected.

In another embodiment of the invention, leaf cells are initially treated with high auxin media, followed by transformation with the transforming DNA and culturing in the presence of high cytokinin levels and a predetermined
30 selection agent. Cells containing transformed plastids are maintained in the presence of the selection agent facilitating the obtention of homoplasmic cells which can then be regenerated into transplastomic plants.

Thus, the present invention provides novel methods
35 and compositions for creating transplastomic plants. The genus *Arabidopsis* belongs to the mustard or crucifer family (*Brassicaceae* or *Cruciferae*), a widely distributed

family of approximately 340 genera and 3350 species. The family is of significant economic importance as a source of vegetable crops, oil seeds, spices and, to a lesser extent, ornamentals. Much of its agricultural importance
5 derives from the genus *Brassica*. Examples for *Brassica* ssp. of economic importance are: *Brassica napus* (oil seed), *Brassica juncea* (oil seed), *Brassica campestris* (oil seed), *Brassica juncea* (oil seed), *Brassica oleracea* (broccoli, cauliflower, cabbage) *Brassica nigra*
10 (black mustard) and *Brassica hirta* (white mustard).

Plastid transformation in *Arabidopsis thaliana* a model species for plant research (Meyerowitz and Sommerville, 1994) and *Brassica* ssp., an important agricultural crop is exemplified herein. These methods
15 are suitable for transformation of plastids in other plants from the *Cruciferae* family.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic drawing illustrating the
20 integration of *aadA* into the *Arabidopsis* plastid genome (ptDNA) after transformation with plasmid pGS31A. Figure 1A shows a map of the transformation vector pGS31A, the ptDNA region containing the integrated spectinomycin resistance (*aadA*) gene (T-ptDNA) and the cognate region
25 of the wild-type ptDNA. 16SrDNA, *rps12/7* and *trnV* are plastid genes (Shinozaki et al., 1986). Figure 1B shows the regions of ptDNA contained in the P1 and P2 probes. Figure 1C is an autoradiogram showing the results of Southern blot hybridization confirming integration of
30 *aadA* in the plastid genome. The P1 targeting sequences hybridize to a 2.72-kb fragment in the wild-type (At) plants and to a larger, 3.82-kb fragment in the transplastomic line (At-pGS31A-16). Note absence of wild-type fragment in transplastomic line. The *aadA*
35 probe, P2, hybridizes only to the larger transplastomic fragment.

Figure 2 is a schematic diagram of the plastid transformation protocol used for *Arabidopsis* leaves.

Figure 3 is a schematic diagram of the different protocols used for obtaining fertile *Arabidopsis* plants from cotyledonary explants of *Arabidopsis thaliana* (RLD) having transformed plastids.

Figure 4 is a map of the plastid targeting region of pGS7 and pGS85A plasmids. Note unique HincII cloning site in plasmid pGS7 and KpnI restriction site in plasmid pGS85, and chimeric kan kanamycin resistance gene. The plastid genes *trnV*, *16SrDNA* and *rps12/7* are described in Shinozaki et al., 1986. Site and direction of transcription initiation is indicated by horizontal arrow.

Figure 5 is a sequence of the targeting region of plasmid pGS7. The genes conferring resistance to kanamycin or spectinomycin will be inserted into the marked Hinc II site .

Figure 6 is a sequence of the plastid targeting region of plasmid pGS31A.

Figure 7 is the sequence of the plastid targeting region of plasmid pGS85A.

DETAILED DESCRIPTION OF THE INVENTION

Insofar as it is known, plastid transformation has been demonstrated in tobacco only. A protocol for the transformation of plastids in *Arabidopsis thaliana* and *Brassica napus* has now been developed and the methods utilized to create these transformants are set forth below. The use of *Arabidopsis* and *Brassica* in the following examples is meant to be illustrative of the methods of the invention. The methods disclosed herein

may be adapted to other plants in the *Cruciferae* family.

The plastids of *Arabidopsis thaliana* have been transformed following biolistic delivery of transforming DNA into leaf cells on the surface of microscopic (1 μ m) tungsten particles as described below in Example I. The transforming plasmid pGS31A, used for these experiments carries a spectinomycin resistance (*aadA*) gene flanked by plastid DNA sequences to target its insertion between *trnV* and the *rps 12/7* operon. Integration of *aadA* by two homologous recombination events via the flanking ptDNA sequences and selective amplification of the transplastomes on spectinomycin medium yielded spectinomycin resistant cell lines. Regenerated plants were homoplasmic in that the plastid genome copies had been uniformly altered by the transforming DNA. The efficiency of plastid transformation was low, two in 201 bombarded leaf samples. However, none of the 98 plants regenerated from the two lines were fertile.

These fertility problems were likely attributable to extended periods of treatment with 2,4-D, an auxin (Van der Graaff and Hooykas, 1996). It is possible that shortening exposure time to this agent may overcome the fertility problem. The relatively long growth period of *Arabidopsis thaliana* to provide a suitable source of leaves for transformation also makes leaves a less desirable tissue source.

Cotyledons and leaves each contain an abundant number of plastid genome copies per cell. Additionally, cotyledons provide a more available tissue source. Accordingly, cotyledon cells have been utilized as recipients for transforming DNA as set forth in Example II below. Cotyledon cells are preferred over leaf cells for practicing the methods of the present invention due to the relatively short (7 days) culturing period to prepare the cells for bombardment with transforming DNA. Another advantage to using cotyledon cells as the target cell is the reported regeneration of fertile *Arabidopsis*

plants from immature cotyledons in the absence of 2,4-D (Patton and Meinke, 1988). In addition, protocols have been described for the regeneration of fertile *Arabidopsis* plants from leaf explants, also in the absence of 2,4-D (Lloyd et al., 1986; Van der Graaff and Hooykas, 1996).

As described in Example III, *Arabidopsis thaliana* and *Brassica napus* belong to the same family, *Cruciferae*, and therefore the plastid genomes share a high degree of homology and are essentially identical (Palmer et al., 1994). Accordingly, plastid transformation vectors and expression cassettes developed for *Arabidopsis* can be used for plastid transformation and expression of foreign genes in *Brassica* species without modification.

The following definitions are provided to facilitate an understanding of the present invention:

Heteroplasmic: refers to the presence of a mixed population of different plastid genomes within a single plastid or in a population of plastids contained in plant cells or tissues.

Homoplasmic: refers to a pure population of plastid genomes, either within a plastid or within a population contained in plant cells and tissues. Homoplasmic plastids, cells or tissues are genetically stable because they contain only one type of plastid genome. Hence, they remain homoplasmic even after the selection pressure has been removed, and selfed progeny are also homoplasmic. For purposes of the present invention, heteroplasmic populations of genomes that are functionally homoplasmic (i.e., contain only minor populations of wild-type DNA or transformed genomes with sequence variations) may be referred to herein as "functionally homoplasmic" or "substantially homoplasmic." These types of cells or tissues can be readily purified to homoplasmy by continued selection on the non-lethal selection medium. Most seed progeny of such plants are homoplasmic in the absence of selection

pressure, due to random sorting of plastid genomes.

Plastome: the genome of a plastid.

Transplastome: a transformed plastid genome.

Transformation of plastids: stable integration of
5 transforming DNA into the plastid genome that is
transmitted to the seed progeny of plants containing the
transformed plastids.

Selectable marker: the term "selectable marker"
refers to a phenotype that identifies a successfully
10 transformed organelle, cell or tissue, when a gene or
allele encoding the selectable marker is included in the
foreign DNA used for transformation.

Transforming DNA: refers to homologous DNA, or
heterologous DNA flanked by homologous DNA, which when
15 introduced into plastids replaces part of the plastid
genome by homologous recombination.

Targeting segment: refers to those homologous
flanking regions which facilitate homologous
recombination between foreign DNA and the plastid genome.

Translationally fused: refers to two coding DNA
20 segments within a construct derived from different
sources spliced together in a construct such that a
chimeric protein is expressed.

High auxin culture medium: plant tissue culture
25 medium which contains auxin only, or a combination of
high concentrations of auxin and very low concentrations
of cytokinins. The response of a plant cell to an auxin
is specific for a given taxonomic group. When different
auxins are applied in combination, their effects may not
30 be additive. Furthermore, the tissue response to auxin
may be modified by cytokinins. Accordingly, the type and
concentration of auxin used should be determined
empirically for the species to be transformed. A
preferred example of a high auxin medium for use in the
35 present invention is C1 medium, containing 1mg/ml of the
auxin 1-napthtaleneacetic acid (NAA) and a low
concentration (0.2 mg/ml) of the cytokinin 6-

benzylaminopurine (BAP). Other auxins, such as indole-3-acetic acid (IAA) and dichloro-phenoxyacetic acid (2,4-D) may also be used to stimulate uniform cell division.

High cytokinin culture medium: like high auxin media, the response of plant cells to high cytokinin media is taxonomic group specific. An example of a preferred high cytokinin medium for use in the present invention is C medium, containing 1 mg/L of BAP, 2 mg/l of 2iP, (6-(gamma,gamma-Dimethylallylamino)purine or IPA, N6-(Isopentenyl)adenine) and a low concentration of the auxin NAA (0.1 mg/L). Other cytokinins which may be used is 6-Furfurylaminopurine (KIN or kinetin).

The detailed description provided in the following examples relates to preferred methods for making and using the DNA constructs of the present invention and for practicing the methods of the invention. Any molecular cloning and recombinant DNA techniques not specifically described are carried out by standard methods, as generally set forth, for example in Sambrook et al., "DNA Cloning, A Laboratory Manual," Cold Spring Harbor Laboratory, 1989 or Ausubel et al. eds. in "Current Protocols in Molecular Biology", John Wiley and Sons, 1995.

The following examples are provided to more fully describe the instant invention. They are not intended to limit the scope of the invention in any way.

EXAMPLE I

Plastid Transformation in Arabidopsis Leaves by Selection for Spectinomycin Resistance

The following materials and protocols enable the practice of the methods of Example I. A schematic diagram of the methods utilized is provided in Figure 2.

Plant material

As the recipient for transformation, the *Arabidopsis* ecotype RLD was used. This ecotype has been

reported to regenerate readily in culture (Marton and Browse, 1991).

5 Construction of vector pGS31A

 The Arabidopsis plastid transformation vector pGS31A is shown in Figure 1. The immediate progenitor of pGS31A is plasmid pGS7, a pBluescript KS(+) phagemid vector (Stratagene) derivative. Plasmid pGS7 carries a 2-kb
10 HindIII-EcoRI Arabidopsis ptDNA fragment containing the 5'-end of the 16S rRNA gene, trnV and part of the rps12/7 operon. During construction of the pGS7 plasmid the HindIII site has been removed by digestion with HindIII (site in 16SrDNA) and KpnI (in vector, treated with the
15 T4 DNA polymerase to remove the single-stranded overhangs) and ligating the blunt ends. Vector pGS31A carries the selectable spectinomycin resistance gene, (Prn::aadA::TpsbA) present in plasmid pZS197 (Svab and Maliga, 1993). The aadA coding region is transcribed from
20 a synthetic promoter consisting of the promoter of the tobacco rRNA operon fused with a synthetic ribosome binding site (Prn). The aadA mRNA is stabilized by transcriptionally fusing sequences downstream of the coding region with the 3'-untranslated region of the
25 tobacco plastid psbA gene (TpsbA). The gene in pGS31A derives from a modified progenitor of pZS197 in which the XbaI site between aadA and TpsbA was removed by blunting. Plasmid pGS31A was obtained by excising the chimeric aadA gene with Eco136II (an isochisomer of SacI, yields blunt
30 ends) and BspHI (single-stranded overhang filled in to obtain blunt ends) for ligation into the unique HincII site of plasmid pGS7 between trnV and the rps12/7 operon.

Tissue culture media

35 The tissue culture protocols were adopted from Marton and Browse (1991) and Czako et al. (1993). The Arabidopsis tissue culture media (ARM) are derivatives of

the Murashige & Skoog (1962) MS medium. ARM medium: MS salts, 3% sucrose, 0.8% TC agar, 2 ml/L of the vitamin solution (100 mg myo-inositol, 5 mg vitamin B1, 0.5 mg vitamin B6, 0.5 mg nicotinic acid, 1 mg glycine and 0.05 mg biotin per ml). ARMI medium: ARM medium containing 3 mg indoleacetic acid (IAA), 0.15 mg 2,4-dichlorophenoxyacetic acid (2,4-D), 0.6 mg benzyladenine (BA) and 0.3 mg isopentenyladenine (IPA) per liter. ARMIr medium: ARM medium supplemented with 0.2 mg/L naphthaleneacetic acid (NAA) and 0.4 mg/L IPA. *Arabidopsis* shoot induction (ASI-N1B1) medium: ARM medium supplemented with 1 mg/L NAA and 1 mg/L BAP. The *Arabidopsis* shoots were rooted on ARM medium. *Arabidopsis* seed culture (ARM5) medium: ARM medium supplemented with 5% sucrose. The stocks of plant hormones were filter sterilized, and added to media cooled to 45 °C after autoclaving.

Selective media contained 500 mg/L spectinomycin HCl and/or streptomycin sulfate. The antibiotics (filter sterilized) were added to media cooled to 45 °C after autoclaving.

Cultivation of *Arabidopsis* plants in sterile culture

For surface sterilization, seeds (25 mg) were treated with 1 ml of commercial bleach (5.25% sodium hypochlorite) in an Eppendorf tube for 5-7 minutes with occasional vortexing. The seeds along with the bleach were poured into a 15 ml conical centrifuge tube containing 10 ml 90% ethanol and incubated for 5-7 minutes. The ethanol-bleach mix was decanted, and the seeds were washed 4 times with 10 ml autoclaved deionized water and finally resuspended in sterile water (approximately 150 seeds/ml). The resulting seed suspension (2 ml) was poured into 10 cm deep (10 mm high) petri dishes containing 50 ml ARM5 medium. The seeds were spread evenly by swirling the suspension. The water was

then removed from the plates by pipetting. The seeds germinated after a 10-15 day incubation at 24°C during which the plates were illuminated for 8 hours using cool-white fluorescent tubes (2000 lux).

5 To grow plants with larger leaves, seedlings were individually transferred to ARM5 plates (10 plants per 10 cm petri dish) and illuminated for 8 hours with cool-white fluorescent bulbs (lux; 21°C day and 18°C night temperature). The thick, dark green leaves, 1 cm
10 to 2 cm in size, were harvested for bombardment after 5-6 weeks.

Transformation and selection of spectinomycin resistant lines

15 Leaves (approximately 1.5 to 30 mm in length) for plastid transformation were harvested from aseptically grown plants. To cover a circular area 4 to 5 cm in diameter, 12 to 18 leaves were placed on agar-solidified ARMI medium. The pGS31A vector DNA was
20 introduced into leaf chloroplasts by the biolistic process, on the surface of microscopic (1 μ m) tungsten particles using a helium-driven PDS1000 biolistic gun. Fresh leaves were bombarded at 450 psi (target placed at 9 cm from rupture disk; position No. 3 from top in the
25 biolistic gun). Leaves cultured for 4 days on ARMI medium were bombarded at 1100 psi (target placed at 12 cm from rupture disk; position No. 4 from top in the biolistic gun).

 Leaf bombardment was performed in ARMI medium.
30 Following bombardment, the leaves were incubated for two additional days in ARMI medium. After this time period, the leaves were stamped with a stack of razor blades to create a series of parallel incisions 1 mm apart. It has been observed previously that mechanical wounding is
35 essential to induce uniform callus formation in the leaf blades. The stamped leaves were transferred onto the same medium (ARMI) containing spectinomycin (500 mg/ml)

to facilitate preferential replication of plastids containing transformed ptDNA copies. The ARMI medium induces division of the leaf cells and formation of colorless, embryogenic callus. After 7-10 day selection on ARMI medium, spectinomycin selection was continued on the ARMIr medium which normally induces greening. Since spectinomycin prevents greening of wild-type cells, only spectinomycin-resistant cells formed green calli. Visible green cell clusters on the selective ARMIr medium appeared within 21 to 70 days.

In 201 bombarded samples 19 spectinomycin-resistant lines were obtained. Plant regeneration was attempted in 14 spectinomycin-resistant lines, and succeeded in 10 of them. Shoots from the green calli regenerated on the ASI-N1B1 medium, and were rooted on ARM medium.

Table 1 sets forth the recovery of spectinomycin resistant cell lines after biolistic delivery of plasmid pGS31A.

Table 1. Recovery of spectinomycin resistant lines after bombardment of *A. thaliana* with plasmid pGS31A

DNA ¹	Number of Samples	psi ²	Number of Spc ³ Plant		Transgenic pt Nucleus		Spont. mutant.
N/A	100		1	0			1
pGS31A	40	1100	8	6	1	7	0
pSG31A	151	450	11	8	1	10	0

¹The control plates were not bombarded.

²psi=pounds per square inch, the value of reapture disk.

Southern hybridization analysis of total cellular DNA to verify plastid transformation

Spectinomycin resistance may be due to expression of *aadA* in plastids (Svab and Maliga, 1993), expression of *aadA* in the nucleus (Svab et al., 1990b), or spontaneous mutation (Fromm et al., 1987; Svab and Maliga, 1991). Southern hybridization was performed to identify plastid transformants in the spectinomycin

resistant lines isolated. Total cellular DNA was isolated according to Mettler (1987). Restriction enzyme-digested DNA was electrophoresed in 0.7 % agarose gels and transferred to nylon membrane (Amersham) using the
5 PosiBlot transfer apparatus (Stratagene). Blots were probed by using Rapid Hybridization Buffer (Amersham) with ³²P labeled probes generated by random priming (Boehringer-Mannheim). When using the targeting ptDNA as a probe, in lines At-pGS31A-2 and At-pGS31A-16, the 3.82-
10 kb transgenic fragment was the only fragment detected indicating that the wild-type ptDNA copies have been selectively diluted out during cell divisions on the selective medium. The same transgenic fragment also hybridized with the *aadA* probe (Figure 1C).

15 Among the 19 spectinomycin resistant lines 17 nuclear transformants were identified by a wild-type fragment on Southern blots when hybridizing with the targeting ptDNA probe. Note that the Southern blots used were optimized for the high-copy (10,000 per cell) leaf
20 ptDNA and will not give a signal with a few nuclear *aadA* copies.

Spontaneous mutants are expected to have wild-type ptDNA targeting fragment on Southern blots and no PCR-amplifiable *aadA* gene. In the sample of 19
25 spectinomycin resistant lines, no such spontaneous mutant was found.

PCR amplification of inserted *aadA* sequences

30 DNA was amplified according to standard protocols (1 min at 92 °C, 1.5 min at 58 °C, 1.5 min at 72 °C, 30 cycles). Spectinomycin resistance being to result of *aadA* expression may be verified by PCR amplification of an 407 nucleotide internal segment using the following primers:
35 5'-GCTTGATGAAACAACGCGG-3'
5'-CCAAGCGATCTTCTTCTTGTC CAAG-3'

Transplastomic Arabidopsis Plants

While the transplastomic *Arabidopsis* plants all flowered, none of them set seed after selfing, or after fertilization with pollen from wild-type plants. Included
5 among these were 98 plants regenerated from the two lines in which spectinomycin resistance was due to plastid transformation, and 66 plants regenerated from 12 lines in which spectinomycin resistance was due to expression of *aadA* in the nuclear genome.

10

Conclusions and Implications

An important agricultural breakthrough, plastid transformation in the model species *Arabidopsis thaliana* is described in the instant invention. Based on the
15 foregoing results, it has been found that a chimeric *aadA* gene, when inserted in the *Arabidopsis* ptDNA targeting cassette, was suitable to recover plastid transformants following biolistic delivery of the transforming DNA. However, the number of *Arabidopsis* plastid transformants
20 was significantly lower, about one in 100, than anticipated based on the transformation of tobacco plastids which yields on average one transformant per bombarded sample (Svab and Maliga, 1993; Zoubenko et al., 1994). There may be multiple reasons for the relatively
25 low transformation efficiency. Inherent species-specific differences, such as relatively inefficient homologous recombination system in *Arabidopsis* chloroplasts could be one obvious reason.

In tobacco vector pZS197, *aadA* is flanked by
30 1.56-kb and 1.29-kb of ptDNA, and yields ~ 1 transformant per bombardment (Svab et al., 1993). In plasmid pRB15, also a tobacco vector, *aadA* is flanked by larger targeting segments, 1.56-kb and 3.6-kb of ptDNA, and yields approximately 5 plastid transformants per
35 bombardment (Bock and Maliga, 1995). In *Arabidopsis* vector pGS31A *aadA* is flanked only by approximately 1-kb plastid targeting sequence on both sides. Therefore, the

efficiency of plastid transformation in *Arabidopsis* may be significantly improved by increasing the size of the targeting ptDNA fragment.

5 In contrast to tobacco, in which most of the plants regenerated from leaves are fertile, it was surprising to find that none of the 164 regenerated *Arabidopsis* plants set seed. Lack of fertility, in part, may be due to the extensive polyploidy of leaf tissue as reported by Galbright et al., (1991) and Melaragno et al.
10 (1993). An additional reason for lack of fertility may be the prolonged exposure of the cultures to 2,4 D (Van der Graaff and Hooykaas, 1996).

EXAMPLE II

Plastid Transformation in *Arabidopsis*

15 Cotyledons by Selection for Kanamycin Resistance

Plastid transformation has been obtained in *Arabidopsis thaliana* by selection for spectinomycin resistance in leaf cultures following bombardment with DNA-coated tungsten particles, as set forth in Example I.
20 While plastid transformation has been successful, the regenerated plants were not fertile. These obstacles have been overcome by altering certain parameters of the transformation protocol.

The protocol developed and set forth in this
25 Example has the following salient features: (1) Cotyledons obtained by germinating mature seed are used to advantage because of their ready availability, and the ease by which large quantities of sterile cotyledons are obtained from surface-sterilized seed. (2) The protocol
30 has two distinct steps. The first step employing a high auxin medium to induce uniform cell division throughout the cotyledon (Stage I) and the second step including a high cytokinin medium to induce plant regeneration (Stages II and III). The protocol was designed to either
35 minimize exposure to medium containing 2,4-D during tissue culture, or more preferably to eliminate such exposure completely. (3) Initial culturing of the

cotyledon cells at a high density, i.e., 500-200 cotyledons/ 20ml in liquid culture medium during the first 8 days (Stage I, II) proved essential for obtaining abundant plant regeneration later.

5 The protocol for plastid transformation in *Arabidopsis* utilizing cotyledons as target tissue and kanamycin-resistance as a selective marker was implemented as follows. The chimeric kan gene derives from plasmid pTNH7, a pUC118 derivative encoding
10 neomycin phosphotransferase (NPTII), an enzyme which enzymatically inactivates the kanamycin antibiotic. The same chimeric kan gene in a tobacco targeting plasmid (plasmids pTNH32) was used for direct selection of plastid transformants in tobacco (Carrer et al. 1993)
15 The construction of the kan gene was described in more detail in this same reference. Plasmid pGS85A was obtained by excising kan from pTNH7 as a SacI/PstI fragment, blunting, and cloning the fragment into the HincII site of plasmid pGS7 (Figure 4). The kan gene in
20 pGS85A, as *aadA* in plasmid pGS31A, is expressed in a *Prn/TpsbA* cassette. However, the five N-terminal amino acids of the highly-expressed *rbcl* coding region were translationally fused with the neomycin
25 phosphotransferase N-terminus. This translational fusion in tobacco lead to the accumulation of NPTII at 10X higher levels than from identical constructs without the *rbcl* N-terminal segment. The DNA sequence of pGS85A, including that of the chimeric kan gene, is set forth herein.

30 Initially, seed-set was tested in plants regenerated via the tissue culture protocol. Selection of kanamycin resistant clones after bombardment with DNA-coated tungsten was subsequently assessed. These improvements to the method are suitable for the
35 generation of fertile, transformed *Arabidopsis* plants. The following material and protocols were utilized in practicing the methods of this Example II.

Seed germination

Seeds of *Arabidopsis thaliana* ecotype RLD are surface sterilized using commercial bleach (5 % sodium hypochlorite) for 5 minutes followed by a subsequent 5 minute treatment with 95% ethanol. A drop of Triton X-100 was added to the bleach to wet the surface of the seeds during the sterilization period. After sterilization, seeds were washed 5-6 times with sterile deionized water. Seeds were germinated on GM medium in 10 cm Petri dishes. See Table 2. The Petri dishes were incubated for 8 to 9 days in a Percival growth chamber at 23°C under continuous light.

Table 2. Composition of seed germination (GM) medium.

<u>Medium</u>	<u>Concentration (mg/L)</u>
MS basal salts	0.5 X
myo-inositol	100
Thiamine	0.1
Pyridoxine	0.5
Nicotinic acid	0.5
Glycine	2.0
Sucrose	30 g/L
pH	5.8

Reference: van der Graaff and Hooykaas, 1996. .

Tissue culture media and culture conditions

Compositions of the tissue culture media used for Stages I, II and III of the selection protocol are listed in Tables 2 and 3. Stage I and Stage II liquid cultures were established by aseptically transferring at least 50 to 2000 cotyledons to a Petri dish (100 mm x 20 mm), each dish containing approximately 20 ml of medium. The Petri dishes were incubated at 23°C on a New Brunswick G10 gyrotory shaker at 60 rpm and illuminated for 16 hours with cool fluorescent light. In the Stage III protocol, cotyledons were incubated on agar-solidified (0.8 % TC agar, JRH Biosciences) media at approximately 25-30 cotyledons per Petri dish (100 mm x 20 mm) in 50 ml of media. The cultures were illuminated as described for Stages I and II.

Regenerated plants were directly transferred to

GM medium in Magenta boxes with vented lids for gas exchange. Plants in the Magenta boxes were incubated in the culture room at 23°C, and illuminated for 16 hours with cool fluorescent light. The plants flowered and set
5 seeds in the boxes.

The methods described for Example I were modified to generate fertile *Arabidopsis* plants having transformed plastid genomes. Three distinct tissue culture stages were employed to obtain plastid transformation. Stage I:
10 liquid culture, in high auxin medium to stimulate uniform cell division. Stage II: liquid culture, in high cytokinin medium to induce plant regeneration from the transformed cells. Stage III: culture on agar-solidified medium, containing high levels of cytokinins also to
15 induce plant regeneration.

A schematic diagram of the strategy used to identify the best protocol for obtaining fertile plastid transformants is outlined in Figure 3. To induce uniform cell division in liquid culture, four media, C1 (van der
20 Graaff and Hooykaas, 1996), ARM I (Marton and Browse, 1991), R3 and PG1 (Feldmann and Marks, 1986; reported to induce callus and/or somatic embryogenesis in *Arabidopsis*) were utilized. Stage I treatment was kept short (2 days) to adopt to the usual timing of
25 transferring the explants to a selective medium after bombardment, and to minimize the adverse effect of 2-4-D, if used at all. The composition of the Stage I tissue culture media utilized is set forth in Table 3 below.

Table 3: Composition of Stage I tissue culture media*.

30	Media	ARM I	C1	R3	PG1
	Basal salts	MS	MS	MS	MS
	Vitamins	ARM I	B5	MS	B5
	2,4-D	0.15	-	0.5	2.2
	BAP	0.6	0.2	-	-
35	IAA	3.0	-	5.0	-
	IPA	0.3	-	-	-
	NAA	-	1.0	-	-
	KIN	-	-	0.3	0.05
	Sucrose	30g	30g	30g	30g
40	pH	5.8	5.8	5.8	5.8

*All components are in mg/L. References: ARM1, Marton and Browse, 1991; C1, van der Graaff and Hooykaas, 1996; R3 and PG1, Feldmann and Marks (1986).

For Stage II culture, only one medium (A; Table 4) was used. This medium was efficient for inducing plant regeneration from immature cotyledons (Patton and Meinke, 1988). The cotyledons at Stage II were kept for a total of 6 additional days at high density in liquid culture.

For Stage III culture, the cotyledons were transferred to four types of agar-solidified regeneration media. These include the A medium developed for plant regeneration from immature embryos (Patton and Meinke, 1988); the B medium developed for plant regeneration from root explants (ARMII; Marton and Browse, 1991); the C medium that designed herein; and the D medium which is an embryo-induction medium for roots (ARMI; Marton and Browse, 1991) and leaf explants (Example I).

Table 4: Stage II and Stage III plant regeneration media.

Media	A medium*	B medium*	C medium*	D medium*
Basal salts	MS	MS	MS	MS
Vitamins	B5	B5	B5	B5
NAA	0.1	-	0.1	-
IAA	-	0.1	-	3.0
BAP	1.0	-	1.0	0.6
2iP	-	4.0	2.0	0.3
2,4-D	-	-	-	0.15
Sucrose	30 g	30 g	30 g	30 g
Agar (TC)	7 g	7 g	7 g	7 g
pH	5.8	5.8	5.8	5.8

*All components are in mg/L. A medium is based on Patton and Meinke, 1988; B medium is the same as ARMII in Marton and Bowse, 1991; C medium developed herein, based on A and D media; D medium is the same as ARMI embryo-induction medium in Marton and Browse, 1991.

Plant regeneration and Testing of Fertility

Cotyledons remained green and slightly expanded in size during the first 2 days of culture at Stage I in all four media. After 2 days in callus/embryo induction medium, cotyledons for Stage II were transferred from all four media to the A liquid regeneration medium. Green callus started appearing after 3 days of culture in A medium and by the 7th day callus appeared all over the cotyledons. At this stage cultures were transferred to

the semi-solid media of Stage III which promotes embryo/shoot growth. Calli derived from media 1 (ARM1) and 2 (C1) were green. Development of plantlets from these explants could be seen by 21 days of culture.

5 Callus derived from media 3 (R3) and 4 (PG1) was also green but very compact. This is probably due to the high concentration of 2,4-D in the Stage I media. A few plantlets in these cultures appeared only after 30 days. Plants from all cultures were transferred to hormone free
10 GM medium as soon as they were 5-10 mm in size.

The protocols diagrammed in Figure 3 were evaluated at two levels: uniform induction of cell division and shoot regeneration from the cotyledons; and by production of viable seed on the regenerated plants.
15 The results are summarized in Table 5. Based on the first criterion, the best combination was 2AC, that is C1 medium at Stage I and C medium at Stage III, these treatments resulted in prolific shoot regeneration which was observed on each of the explants. The second best
20 combination was 1AC (35 out of 40 explants regenerating shoots), with ARM1 at Stage I and medium C at Stage III. Combinations with media 3 and 4 at Stage I performed very poorly, with only a very small fraction of cotyledons forming shoots.

25 As to formation of viable seed, with one exception each of the regenerated plants produced viable seed. See Table 5. Most importantly, no adverse effect on fertility was found in the two combinations (2AC and 1AC) in which shoot regeneration is prolific.

30

35

Table 5

Seed-set in Magenta boxes on *Arabidopsis thaliana* RLD plants regenerated via plastid transformation protocols schematically shown in Figure 3.

5	Media	Number of cotyledons cultured	Number of cotyledons with shoots	Number of plants in boxes	Number of with viable seed
	1AA	40	20	8	8
10	1AB	40	25	8	8
	1AC	40	35	8	8
	1AD	40	-	-	-
	2AA	40	25	12	12
	2AB	40	22	8	7
15	2AC	40	40	16	16
	2AD	40	2	-	-
	3AA	40	12	4	4
	3AB	40	6	4	4
	3AC	40	20	-	-
20	3AD	40	1	-	-
	4AA	40	1	-	-
	4AB	40	1	-	-
	4AC	40	4	4	4
25	4AD	40	1	-	1

Selection of plastid transformants by kanamycin resistance

Expression of kan encoding neomycin phosphotransferase (NPTII) confers resistance to kanamycin when introduced into the *Arabidopsis* nucleus. Engineered forms of kan have been extensively used to obtain nuclear transformants in *Arabidopsis*, see Valvekens et al., 1988. and *Brassica*, see Radke, et al. 1992. The kan gene has been converted into a plastid marker for the selection of plastid transformants in tobacco (Carrer et al., 1993). As set forth in Example I, *Arabidopsis* plastid transformants have been obtained by selection for spectinomycin resistance conferred by *aadA* in the tobacco Prn/TpsbA cassette. Prn is a promoter derived from the plastid rRNA operon and TpsbA contains the plastid psbA gene 3' untranslated region required for the stabilization of chimeric plastid mRNAs (Svab and Maliga, 1993). A kanamycin resistance marker gene suitable for the selection of plastid transformants may be obtained by expressing kan in the Prn/TpsbA cassette. A suitable kanamycin resistance plastid transformation vector from

Arabidopsis and *Brassica* is the pGS85A vector which carries the chimeric kanamycin gene from plasmid pTNH32 (Carrer et al. 1993). The insertion site in pGS85A is the Hinc II site in the trnV/rps12/7 intergenic region.

5 However, other intergenic regions in the plastid genome may be targeted as in providing the introduced transgene does not interfere with the expression of the flanking plastid genes.

Plastid transformation may be carried out following the 1AC or 2AC tissue culture protocols outlined above. To prepare a suitable target tissue for transformation, cotyledons from 8-9 day old seedlings are cut from seedlings in liquid ARM1 and C1 media and cultured for two days as dictated by the 1AC and 2AC protocols (Figure 3). After two days the cotyledons are transferred to filter paper (Whatman No. 4) on agar-solidified non-selective medium of identical composition. Approximately 50 to 70 cotyledons are required to cover a 3 cm² area. The cotyledons are then bombarded with plasmid pGS85A, a kanamycin resistance, transforming *Arabidopsis* vector. Plasmid preparation, coating of tungsten particles and bombardment should be carried out as described for tobacco (Maliga, 1995). For phenotypic expression, the cotyledons may be left in the same plates for two days. Subsequently, the cotyledons may be transferred to a selective liquid A medium containing 50 mg/L kanamycin sulfate and cultured for an additional seven days. After 7 days, cotyledons are transferred to a selective, agar-solidified C medium containing 50 mg/L kanamycin. In an alternative embodiment, selection may be carried out initially using kanamycin at 25 mg/ml. At later stages of culture, the kanamycin concentration is increased to 50 mg/ml. Callus growth from the transformed cells on the selective medium may be observed as early as one week. However, additional kanamycin-resistant clones may appear for several more weeks. Some of these are plastid

transformants, while others acquire resistance to kanamycin due to the expression of the plastid kan gene in the nucleus (Carrer et al., 1993). The two classes of kanamycin-resistant clones can be readily distinguished

5 DNA gel blot analysis and PCR analysis (as described in Example I). DNA was amplified according to standard protocols (1 min at 92 °C, 1.5 min at 58 °C, 1.5 min at 72 °C, 30 cycles). Kanamycin resistance occurs as the result of neomycin phosphotransferase gene expression

10 which may be verified by PCR amplification of a 548 nucleotide internal segment using the following primers:
5'-CCGACCTGTCCGGTGCCC-3'
5'-CACGACGAGATCCTCGCCG-3'.

15

EXAMPLE III
Plastid Transformation in *Brassica napus* Leaves by selection for Resistance to spectinomycin and kanamycin

20

Given their essentially identical genomes, plastid transformation vectors and expression cassettes developed for *Arabidopsis* can be used to advantage for plastid transformation and expression of foreign genes in

25 *Brassica* species without modification.

Certain plastid expression signals derived from evolutionarily distant species function in *Arabidopsis* and *Brassica* plastids. This observation is supported by the results described in Example I demonstrating that the tobacco Prn/TpsbA cassette can be used for expressing

30 the selectable spectinomycin resistance gene (aadA) in *Arabidopsis* plastids. However, not every tobacco expression signal functions properly in *Arabidopsis*. Studies with a vector identical to PGS31A, except that

35 the termination signal TpsbA, has been replaced with signal Trps16 has a dramatic effect on obtaining plastid transformants. This plasmid gene was obtained by inserting the Prn/Trps16 cassette into targeting site in the pGS7 vector. See figure 4. Zero plastid

transformants were obtained out of 416 samples bombarded with this plasmid. As mentioned above, when a Prn/TpsbA cassette (cassettes described in Staub and Maliga, Plant Journal 6:547-553, 1994 and Svab and Maliga, 1993, the subject matter of which is incorporated herein by reference) was utilized to transform Arabidopsis leaves, plastid transformants were obtained, 2 out of 210 samples bombarded.

Due to their taxonomic relatedness, *Arabidopsis* and *Brassica* species respond similarly in tissue culture to plant hormones or to antibiotics. As a result, plant regeneration from cultured cells and selection of transgenic lines by antibiotic resistance may be accomplished by essentially the same protocol. Both *Arabidopsis* and *Brassica* leaf or cotyledon explants respond to 500 mg/L spectinomycin with prolific callus growth in wild-type, non-transformed tissue on shoot regeneration medium, such as medium C described in Table 6. This response differs significantly in tobacco leaf tissue wherein exposure to 500 mg/ml of spectinomycin results in a severe inhibition of callus proliferation on shoot induction medium. Thus, tobacco plastid (and nuclear gene) transformants can be readily regenerated on a shoot induction medium containing spectinomycin at 500 mg/L (Svab and Maligam 1993). Unfortunately, rapid callus proliferation on spectinomycin-containing C shoot/embryo regeneration medium (see table 6) prevents the recovery of *Arabidopsis* and *Brassica* plastid transformants. Culture conditions must be improved to suppress rapid callus growth to facilitate the recovery of plastid transformants. Such conditions are outlined in Example I. While selection was feasible and plastid transformants were obtained using the methods of Example I, the transplastomic plants generated were not fertile. However, given the higher tolerance of *Brassica* to 2,4 D (Radke et al., 1992) the same protocol described in example I may be adapted for use in *Brassica*.

The data presented in Example II indicate that kanamycin selection is compatible with the regeneration protocols described. Accordingly, kanamycin is the favored antibiotic for the selection of plastid transformants in the *Cruciferae* taxonomic group.

Examples I and II disclose protocols for the regeneration of transgenic plants from *Arabidopsis* leaves and cotyledons. A protocol for the regeneration of transgenic plants in *Brassica* would involve a two-stage protocol (application of two different media) for leaves, and a three-stage protocol (application of three different media) for cotyledons. The three-stage protocol described for use in the plastid transformation of *Arabidopsis* cotyledons in Example II is suitable for use in *Brassica*. Accordingly only the methods for transforming *Brassica* leaf plastids in a two stage process will be described below .

Plastid transformation in *Brassica* utilizing leaves as target tissue and kanamycin resistance as the selective marker

Brassica Stage I culture results in the uniform induction of cell division in leaves or cotyledons. The objective of Stage II is regeneration of transgenic plants. A suitable Stage I medium for the induction of cell division would be the ARMI medium discussed in Examples I and II. Suitable Stage II regeneration media would be the B medium (ARMII in Marton and Browse, 1991), C medium (this study) and E medium (Pelletier et al. 1983) listed in Table 6.

Table 6 Stage II *Brassica* plant regeneration media*

Media	B medium	C medium	E medium
Basal salts	MS	MS	MS
Vitamins	B5	B5	B5
NAA	-	0.1	1.0
IAA	0.1	-	-
BAP	-	1.0	-
2iP	4.0	2.0	1.0
GA3	-	-	0.02
Sucrose	30 g	30 g	30 g
Agar (TC)	7 g	7 g	7 g
pH	5.8	5.8	5.8

*All components are in mg/L. B medium is the same as ARMII in Marton and Bowse, 1991; C medium is this study; E medium is the *cruciferae*

regeneration medium of Pelletier et al. 1983.

For selection of plastid transformants, *Brassica*
5 *napus* cv. Westar seeds should be surface sterilized, and
germinated aseptically in Magenta boxes as described for
Arabidopsis in Example II. After three to four weeks,
the leaves are harvested, and directly placed a Whatman
filter paper placed on agar-solidified non-selective
10 Stage I medium. Following bombardment with DNA of the
appropriate plastid transformation vector carrying a
selectable kanamycin-resistance marker, as described in
Example II, the plates are incubated for two days in the
light (16 hours) at 25°C. After 2 days the leaves are
15 incised with a stack of sterile razor blades, and
transferred to the same Stage I medium supplemented with
50 mg/L of kanamycin sulfate. In an alternative
embodiment, selection may be carried out initially using
kanamycin at 25 mg/ml. At later stages of culture, the
20 kanamycin concentration is increased to 50 mg/ml. After
two weeks on the selective Stage I medium, the leaves are
transferred to one of the Stage II media for plant
regeneration. Kanamycin resistant clones are identified
by their rapid growth and shoot regeneration on the
25 selection medium. Kanamycin resistance may be due to
plastid transformation or integration of the kanamycin
marker gene into the nuclear genome. Plastid
transformation is verified by PCR and DNA gel blot
analysis in tissue samples taken from kanamycin-resistant
30 calli and regenerating shoots. The regenerated shoots
are then rooted and transferred to soil in the greenhouse
following standard protocols.

REFERENCES

1. Bendich, A.J. (1987) Why do chloroplasts and mitochondria contain so many copies of their genome?
5 Bioessays 6, 279-282.
2. Bock, R. and Maliga, P. (1995) In vivo testing of a tobacco plastid DNA segment for guide RNA function in psbL editing. Molec. Gen. Genet. 247, 439-443.
10
3. Carrer, H., Hockenberry, TN, Svab, Z., Maliga, P. (1993) Kanamycin resistance as a selectable marker for plastid transformation in tobacco. Molec. Gen. Genet. 241, 49-56.
15
4. Carrer, H., Maliga, P. (1995) Targeted insertion of foreign genes into the tobacco plastid genome without physical linkage to the selectable marker gene. Biotechnology 13, 791-794.
20
5. Czako, M., Wilson, J. and Marton. L. (1993) Sustained root culture for generation and vegetative propagation of transgenic *Arabidopsis thaliana*. Plant Cell Rep. 12, 603-606.
25
6. Fromm, H., Edelman, M., Aviv, D. and Galun, E. (1987) The molecular basis of basis of rDNA-dependent spectinomycin resistance in *Nicotiana chloroplasts*. EMBO J., 6, 3233-3237.
30
7. Galbright, D.W., Harkins, K.R. and Knapp, S. (1991) Systemic endopolyploidy in *Arabidopsis thaliana*. Plant Physiol. 96, 985-989.
8. Goulds, T., Maliga, P. & Koop, H.U. (1993) Stable plastid transformation in PEG-treated protoplasts of *Nicotiana tabacum*. Bio/Technology, 11, 95-97
35
9. Hajdukiewicz, P., Svab, Z. and Maliga, P. (1994) The small, versatile pPZP family of *Agrobacterium* binary vectors for plant transformation. Plant. Mol. Biol. 25, 989-994.
40
10. Maliga, P. (1993) Towards plastid transformation in flowering plants. Trends Biotechnol., 11, 101-106.
45
11. Maliga, P. (1995) Biolistic transformation of tobacco cells with nuclear drug resistance genes. In Methods in Plant Molecular Biology - A Laboratory Manual, (Maliga, P., Klessig, D., Cashmore, A., Gruissem W. and Varner, J., eds.). Cold Spring Harbor: Cold Spring Harbor Laboratory Press, pp. 37-54.
50
12. Maliga, P., Carrer, H., Kanevski, I., Staub, J., Svab Z. (1993) Plastid engineering in land plants: a conservative genome is open to change. Phil. Trans. R.
55

Soc. Lond. B 341, 449-454.

13. Marton, L. and Browse, J. (1991) Facile transformation of *Arabidopsis thaliana*. Plant Cell Rep. 10, 235-239.
14. McBride, K.E., Svab, Z., Schaaf, D.J., Hogan, P.S., Stalker, D.M., Maliga, P. (1995) Amplification of a chimeric *Bacillus* gene in chloroplasts leads to an extraordinary level of an insecticidal protein in tobacco. Biotechnology, 13, 362-365.
15. Melaragno, J.E., Mehrotra, B. and Coleman, A.W. (1993) Relationship between endopolyploidy and cell size in epidermal tissue of *Arabidopsis*. Plant Cell 5, 1661-1668.
16. Mettler, I.J. (1987) A simple and rapid method for minipreparation of DNA from tissue cultured plant cells. Plant Mol. Biol. Reporter 5, 346-349.
17. Meyerowitz, E.M. and Somerville, C.R. (1994) *Arabidopsis*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.
18. Murashige, T. and Skoog, F. (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant., 15, 493-497.
19. O'Neill, C, Horvath, G.V., Horvath, E., Dix, P.J. and Medgyesy, P. (1993) Chloroplast transformation in plants: polyethylene glycol (PEG) treatment of protoplasts is an alternative to biolistic delivery systems. Plant J., 3, 729-738.
20. Palmer, J.D. (1991) Plastid chromosomes: structure and evolution. In The Molecular Biology of Plastids, Cell Culture and Somatic Cell Genetics of Plants, vol. 7A (L. Bogorad, L. and Vasil I.K. eds.). San Diego: Academic Press, pp. 5-53..
21. Palmer, J.D., Downie, S.R., Nugent, J.M., Brandt, P., Unseld, M., Klein, M., Brennicke, A., Schuster, W. and Borner, T. (1994) Chloroplast and mitochondrial DNAs of *Arabidopsis thaliana*: Conventional genomes in an unconventional plant. In *Arabidopsis*, (Meyerowitz, E.M. and Somerville, C.R., eds.) Cold Spring Harbor: Cold Spring Harbor Laboratory Press, pp. 37-62.
22. Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsubayashi, T., Zaita, N., Chunwongse, J., Obokata, J., Yamaguchi-Shinozaki, K., Ohto, C., Torazawa, K., Meng, B.Y., Sugita, M., Deno, H., Kamoyashira, T., Yamada, K., Kusuda, J., Takawa, F., Kato, A., Tohdoh, N., Shimada, H. and Suguira, M. (1986) The complete nucleotide sequence of the tobacco

- chloroplast genome: its gene organization and expression. EMBO J., 5, 2043-2049.
23. Staub, J. & Maliga, P. (1992) Long regions of homologous DNA are incorporated into the tobacco plastid genome by transformation. Plant Cell 4, 39-45.
24. Svab, Z. and Maliga, P. (1991) Mutation proximal to the tRNA binding region of the *Nicotiana* plastid 16S rRNA confers resistance to spectinomycin. Molec. Gen. Genet. 228, 316-319
25. Svab, Z. and Maliga, P. (1993) High-frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene. Proc. Natl. Acad. Sci. USA, 90, 913-917.
26. Svab, Z. and Maliga, P. (1993) High-frequency plastid transformation in tobacco by selection for a chimeric *aadA* gene. Proc. Natl. Acad. Sci. USA, 90, 913-917.
27. Svab, Z., Hajdukiewicz, P. and Maliga, P. (1990a) Stable transformation of plastids in higher plants. Proc. Natl. Acad. Sci. USA, 87, 8526-8530
28. Svab, Z., Harper, E.C., Jones, J.D.G. and Maliga, P. (1990b) Aminoglycoside-3"-adenyltransferase confers resistance to spectinomycin and streptomycin in *Nicotiana tabacum*. Plant Mol. Biol. 14, 197-205.
29. Valvekens, D., Van Motagu, M. and Van Lijsebettens, M. (1988) Agrobacterium tumefaciens-mediated transformation of *Arabidopsis thaliana* root explants using kanamycin selection. Proc. Natl. Acad. Sci. USA 85, 5536-5540.
30. Zoubenko, O.V., Allison, L.A., Svab, Z. and Maliga, P. (1994) Efficient targeting of foreign genes into the tobacco plastid genome. Nucleic Acids Res., 22, 3819-3824.
31. Feldmann, K.A. and Marks, M.D. (1986) Rapid and efficient regeneration of plants from explants of *Arabidopsis thaliana*. Plant Sci. 47: 63-69.
32. Lloyd, A.M., Barnason, A.R., Rogers, S.G., Byrne, M.C., Fraley, R.T., Horsch, R.B. (1986) Science 234:464-466.
33. Patton, D.A. and Meinke, D.W. (1988) High-frequency plant regeneration from cultured cotyledons of *Arabidopsis thaliana*. Plant Cell Reports 7:233-237.

34. Van der Graaff, E. and Hooykaas, P.J.J. (1996)
Improvements in the transformation of *Arabidopsis*
thaliana. C24 leaf-discs by *Agrobacterium tumefaciens* .
5 Plant Cell Rep. 15: 572-577.
35. Pelletier, G., Primard, C., Vedel, F., Chetrit,
P. Remy, R. Rousselle, R. and Renard M. (1983)
10 Intergeneric cytoplasmic hybridization in cruciferae by
protoplast fusion. Mol. Gen. Genet. 191:244-250.
36. Radke, S. E., Turner, J. C., and Facciotti D. (1992)
15 Transformation and regereation of *Brassica rapa* using
Agrobacterium tumefaciens. Plant Cell Reports 11:499-505.

While certain preferred embodiments of the
20 present invention have been described and specifically
exemplified above, it is not intended that the invention
be limited to such embodiments. Various modifications
may be made to the invention without departing from the
scope and spirit thereof as set forth in the following
25 claims.

What is claimed is:

1. A method for obtaining transplastomic plants, comprising:

5 a) culturing plant cells from said plant in the presence of an agent that stimulates uniform cell division;

b) transferring said plant cells to filter paper on agar-solidified medium;

10 c) delivering to a plastid genome within said plant cells, a transforming DNA, said transforming DNA molecule having:

i) a plurality of targeting segments consisting of plastid DNA sequences from said plastid genome to be transformed, said targeting segments
15 facilitating homologous recombination of said transforming DNA into said plastid genome;

ii) 5' and 3' regulatory sequences derived from plastid DNA operably linked to a selectable marker gene disposed within said targeting segment, said regulatory
20 sequences facilitating expression of the selectable marker gene and stability of mRNA encoded therefrom, said selectable marker gene conferring antibiotic resistance to said plant cells;

iii) 5' and 3' regulatory sequences derived
25 from plastid DNA operably linked to sequences encoding a foreign gene of interest thereby facilitating expression of the foreign gene of interest and stability of mRNA encoded therefrom; and

iv) at least one cloning site for insertion of
30 said foreign gene of interest adjacent to said selectable marker gene, said insertion not interfering with said conferring of said selectable phenotype and function of flanking plastid genes;

d) transferring cells transformed as in step (c)
35 to a culture medium at high density for a predetermined time period; said culture medium containing an agent that promotes continued uniform cell division and plant

regeneration;

e) transferring said cells treated as in step (d) to an agar-solidified culture medium containing said regeneration promoting agent and said antibiotic, said transformed cells being rendered resistant to said antibiotic by expression of said selectable marker gene; and

f) selecting for cells having transformed plastid genomes and inducing plant regeneration therefrom.

10

2. A method as claimed in claim 1, wherein said transforming DNA is delivered by a method selected from the group consisting of biolistic bombardment of said cells with DNA-coated particles, CaPO_4 mediated transfection, electroporation, and polyethylene glycol mediated DNA uptake.

15

3. A method claimed as in claim 1, wherein said plant cells are selected from the group consisting of cotyledon cells, leaf cells, hypocotyls and root cells.

20

4. A method as claimed in claim 1, wherein said selectable marker gene and said foreign gene of interest constitute a monocistronic expression unit.

25

5. A method as claimed in claim 1, wherein said selectable marker gene and said foreign gene of interest constitute a polycistronic expression unit.

30

6. A method as claimed in claim 1, wherein said plastids are chloroplasts.

7. A method as claimed in claim 1, wherein said antibiotic is selected from the group consisting of kanamycin, spectinomycin, and streptomycin.

35

8. A method as claimed in claim 1, wherein said

agent promoting uniform cell division is selected from the group consisting of NAA, IAA, and 2,4-D.

5 9. A method as claimed in claim 1, wherein said regeneration promoting agent is selected from the group consisting of BAP, 21P IPA and KIN.

10 10. A method as claimed in claim 1, wherein said transforming DNA is cloned within vector pGS31A.

 11. A method as claimed in claim 1, wherein said transforming DNA is cloned within vector pGS85A.

15 12. A method as claimed in claim 1, wherein said transforming DNA is cloned within vector PGS7.

 13. A method as claimed in claim 1, wherein said plant is *Arabidopsis thaliana*.

20 14. A method as claimed in claim 1, wherein said plant is *Brassica napus*.

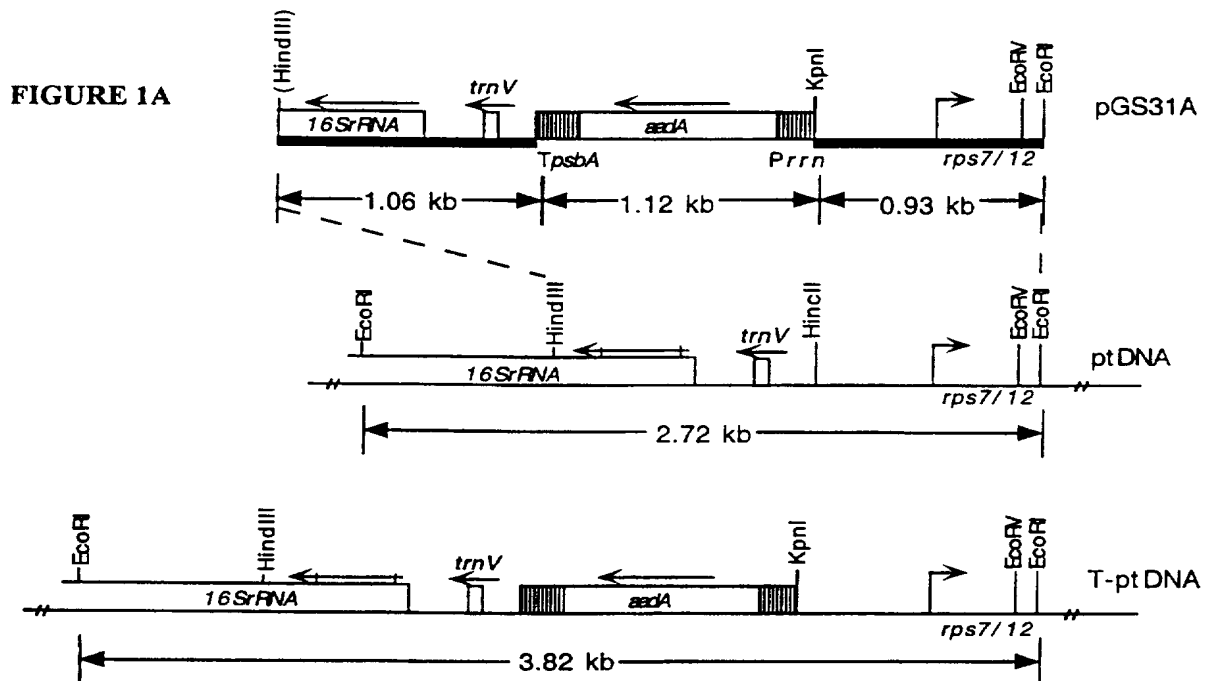


FIGURE 1B

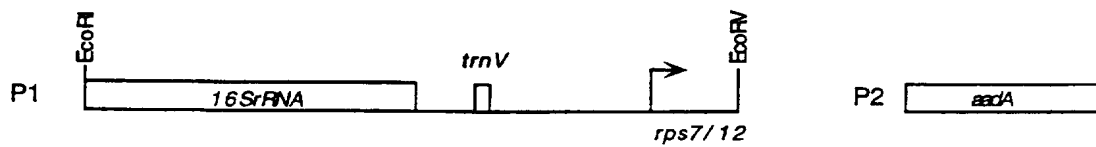
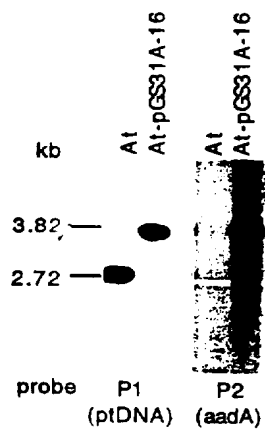


FIGURE 1C



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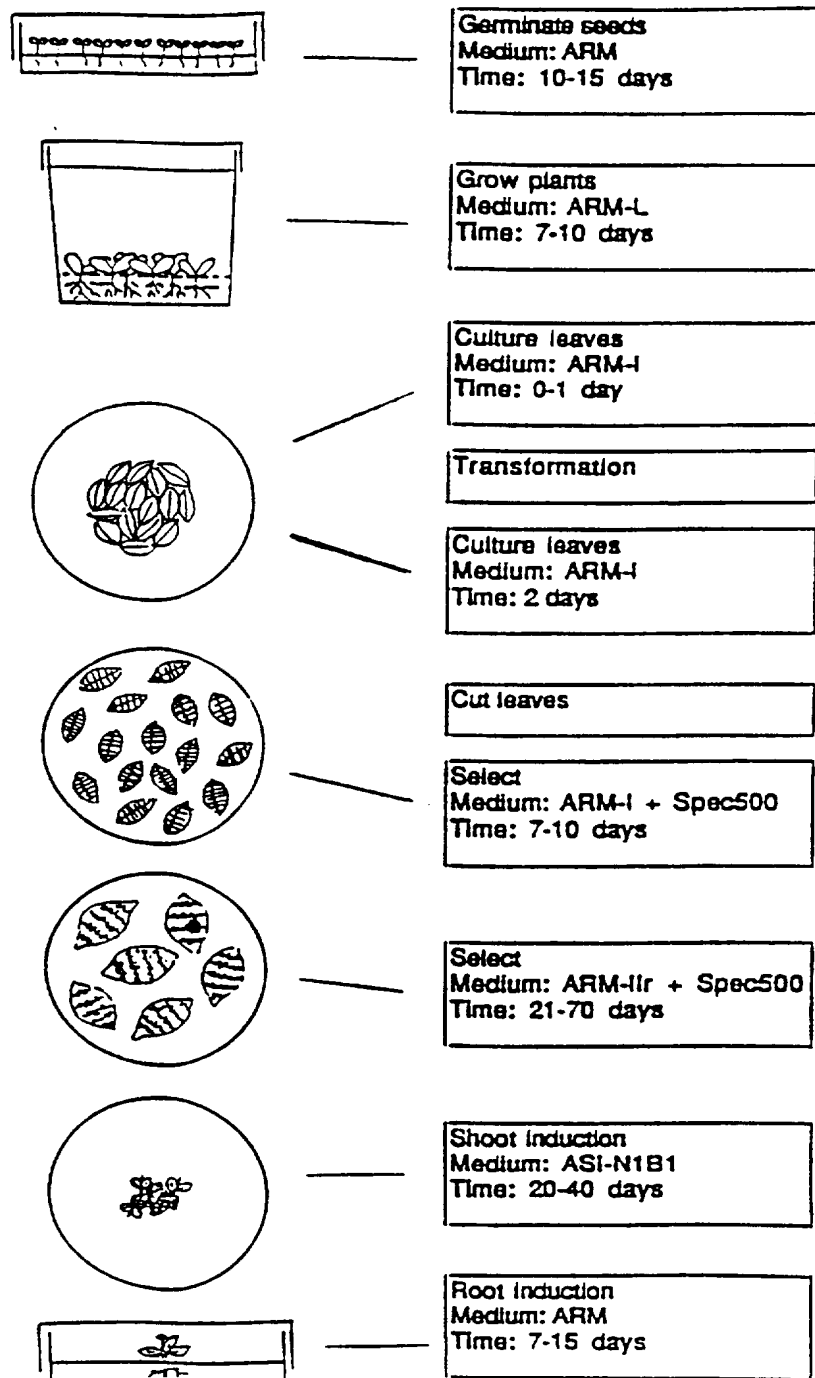
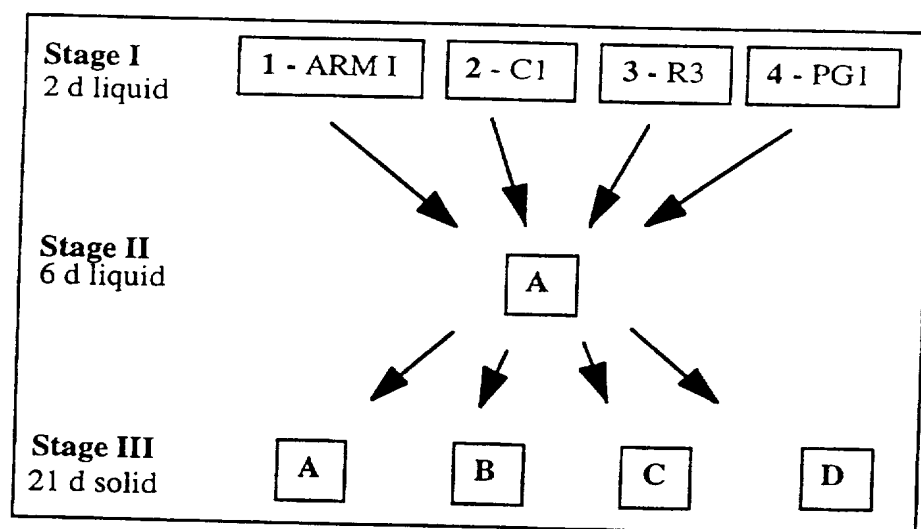


FIGURE 2

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**FIGURE 3**

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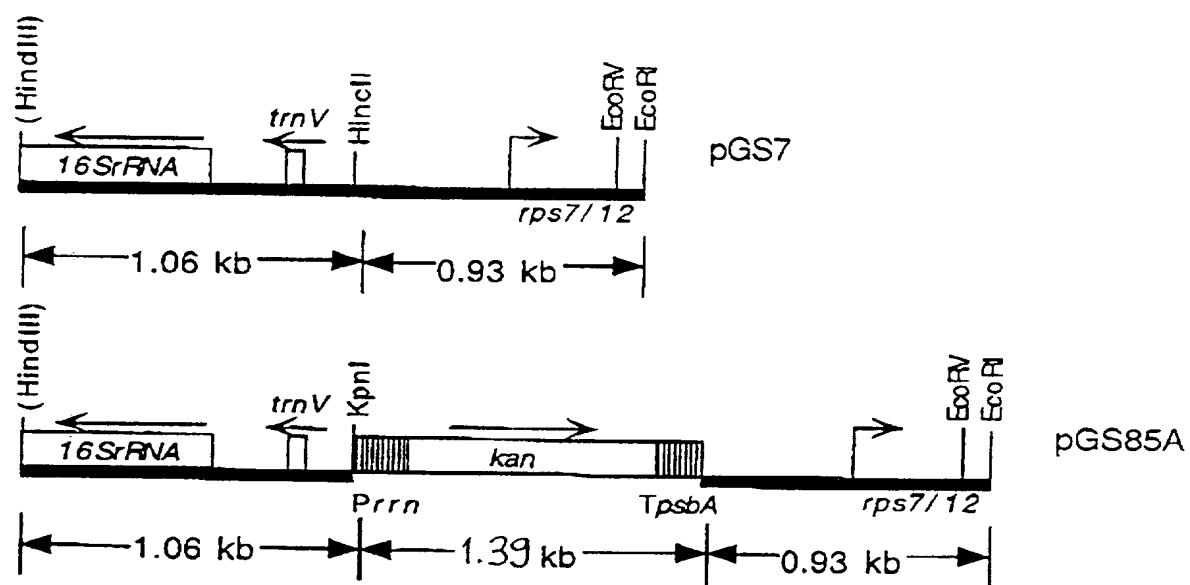


FIGURE 4

HindIII

1 AAGCTTGGTA GTTTCACCG CCTGTCCAGG GTTGAGCCCT GGGATTTGAC
 51 GGCGGACTTA AAAAGCCACC TACAGACGCT TTACGCCCAA TCATTCCGGA
 101 TAACGCTTGC ATCCTCTGTA TTACCGCGGC TGCTGGCACA GAGTTAGCCG
 151 ATGCTTATTC CCCAGATACC GTCATTGCTT CTTCTCTGGG AAAAGAAGTT
 201 CAGGACCCGT AGGCCTTCTA CCTCCACGCG GCATTGCTCC GTCAGGCTTT
 251 CGCCCATTGC GGAAAATTCC CCACTGCTGC CTCCCGTAGG AGTCTGGGCC
 301 GTGTCTCAGT CCCAGTGTGG CTGATCATCC TCTCGGACCA GCTACTGATC
 351 ATCGCCTTGG TAAGCTATTG CCTCACCAAC TAGCTAATCA GACGCGAGCC
 401 CCTCCTCGGG CGGATTCCCTC CTTTGTCTCC TCAGCTACGG GGTATTAGCA
 451 GCCGTTTCCA GCTGTTGTTC CCCTCCCAAG GGNAGGTTCT TACGCGTTAC
 501 TCACcNGTCC GCCACTGGAA ACACCACTTC CCGTCCGACT TGCATGTGTT
 551 AAGCATGCCG CCAGCGTTCA TCCTGAGCCA GGATCGAACT CTCCATGAGA
 601 TTCATAGTTG CATTACTTAT AGCTTCCTTC TTCGTAGACA AAGCTGATTC
 651 GGAATTGTCT TTCATTCCAA GTCATAACTT GTATCCATGC GCTTCATATT
 701 CGCATGGAGT TCGCTCCCAG AAATATAGCT ACCCCTACCC CCTCACGTCA
 751 ATCCCACGAG CCTCTTATCC ATTCTTATTC GATCACAGCG AGGGAGCAAG
 801 TCAAAATAGA AAAACTCACA TTCATTGGGT TTAGGGATAA TCAGGCTCGA
 851 ACTGATGACT TCCACCACGT CAAGGTGACA CTCTACCGCT GAGTTATATC
 901 CCTTCCCCA TCAAGAAATA GAACGACTA ATCCTAAGTC AAAGGGTCGA
 951 GAAACTCAAG GCCACTATTC TTGAACAAC TGGATTGGAG CCGGGCTTTC
 1001 CTTTCGCACT TTATACGGGT ATGAAATGAA AATAATGGAA AAAGTTGGAT
 1051 TCAATTGTCA ACTACTCCTA TCGGAAATAG GATTGACTAC GGATTTCGAGC
 1101 CATAGCACAT GGTTCATATA AACCCTACGA TTCTCCCGAT CTAAATCAAG
 1151 CCGGTTTTAC ATGAAGAAGA TTTGACTCGG CATGTTCTAT TCGATACGGG
 1201 TAGGAGAAAC GGTATTCTTT TCTTAAACTT CAAAAAATAG AGAAATAAGA
 1251 ACCAAGTCAA GATGATACGG ATTAATCCTT TATTCTTGCG CCAAAGATCT
 1301 TCCTATTCCA AGGAACTGGA GTTACATCTC TTTTCCATTT CCATTCAAGA
 1351 GTTCTTATGT GTTTCACcC CCCTTTAAGA cCCGAAAAA TCGACAAATT
 1401 CCCTTTTCTT AGGACCACAT GCGAGATAAC GAAAAAATAA AAGAGAGAAT
 1451 GGTAACCCCA CGATTAACTA TTTTATTTAT GAATTTCTATA GTAATAGAAA
 1501 TACATGTCCT ACCGAAACAG AATTTGTAAC TTGCTATCCT ATCATCTTGC
 1551 CTAGCAGGCA AAGATTTTAC TCCGCGAAAA AGATGATTCA TTCGGATCAA
 1601 CATGAAAGCC CAACTACATT GCCAGAATTT ATATATTGGA AAGAGGTTTA
 1651 CCTCCTTGCT TCTATGGTAC AATCCTCTTC CCGCGGAGCC TCCTTTCTTC
 1701 TCGGTCCGCA GAGACAAAAT GTAGGACTGG TGCCAACAGT TAATCACGGA
 1751 AGAAAGGACT CACTGCGCCA AGATCACTAA CTAATCTAAT AGAATAGAAA
 1801 ATCCTAATAT AATAGAAAAG AAAAGAAGTG TCTTTTCTGA TACTTATGTA
 1851 TACTTTCCCC GGTTCCGTTG CTACTGCGGS TTTACGCAAT TGATCGGATC
 1901 ATCTAGATAT CCCTTCAACA CAACATAGGT CGTCGAAAGG ATCTCGGAGA
 1951 CCCGCCAAAG CACGAAAGCC AGAATCTTTC AGAAAATGAA TTC

HincII*EcoRI*

FIGURE 5

PGS31A derived by inserting Prm::aadA::Tpsba as Eco136II/BspHI
 blunt from pZS179, XbaI site filled in
 PGS31A.seq Length: 1143 March 6, 1997 19:58 Type: N Check: 380

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1      >SEQED (include) reverse of: Prm::aadA::Tpsba as Eco136II/BspHI
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51     CAAAAAAGTC TATGTAAGTA AAATACTAGT AAATAAATAA AAAGAAAAAA

101    AGAAAGGAGC AATAGCACCC TCTTGATAGA ACAAGAAAAT GATTATTGCT

151    CCTTTCTTTT CAAAACCTCC TATAGACTAG GCCAGGATCg ct
      (include) reverse of: povz44a.seq check: 1487 from:
      1954 to: 2898<
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251    attcttccaa ctgatctgcg cgcgaggcca agcgatcttc ttcttgteca

301    agataagcct gtctagcttc aagtatgacg ggctgatact gggccggcag

351    gcgctccatt gccagtcgg cagcgacatc ctteggcgcg attttgccgg

401    ttactgcgct gtaccaaagt cgggacaacg taagcactac atttcgctca

451    tcgccagccc agtcggggcg cgagttccat agcgtaagg tttcatttag

501    cgctcctcaat agatcctgtt caagaaccgg atcaaagagt tctccgccc

551    ctggacctac caaggcaacg ctatgttctc ttgcttttgt cagcaagata

601    gccagatcaa tgcgatcgtt ggctggctcg aagatacctg caagaatgtc

651    attgcgctgc cattctccaa attgcagttc gcgcttagct ggataacgcc

701    acggaatgat gtctgctgct acaacaatgg tgacttctac agcgcgagga

751    atctcgctct ctccagggga agccgaagtt tccaaaaggt cgttgatcaa

801    agctcgccgc gttgtttcat caagccttac ggtaaccgta accagcaaat

851    caatatcact gtgtggcttc aggcggccat ccactgcgga gccgtacaaa

901    tgtacggcca gcaacgtcgg ttcgagatgg cgctcgatga cgccaactac

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Prm::aadA::Tpsba

Figure 6

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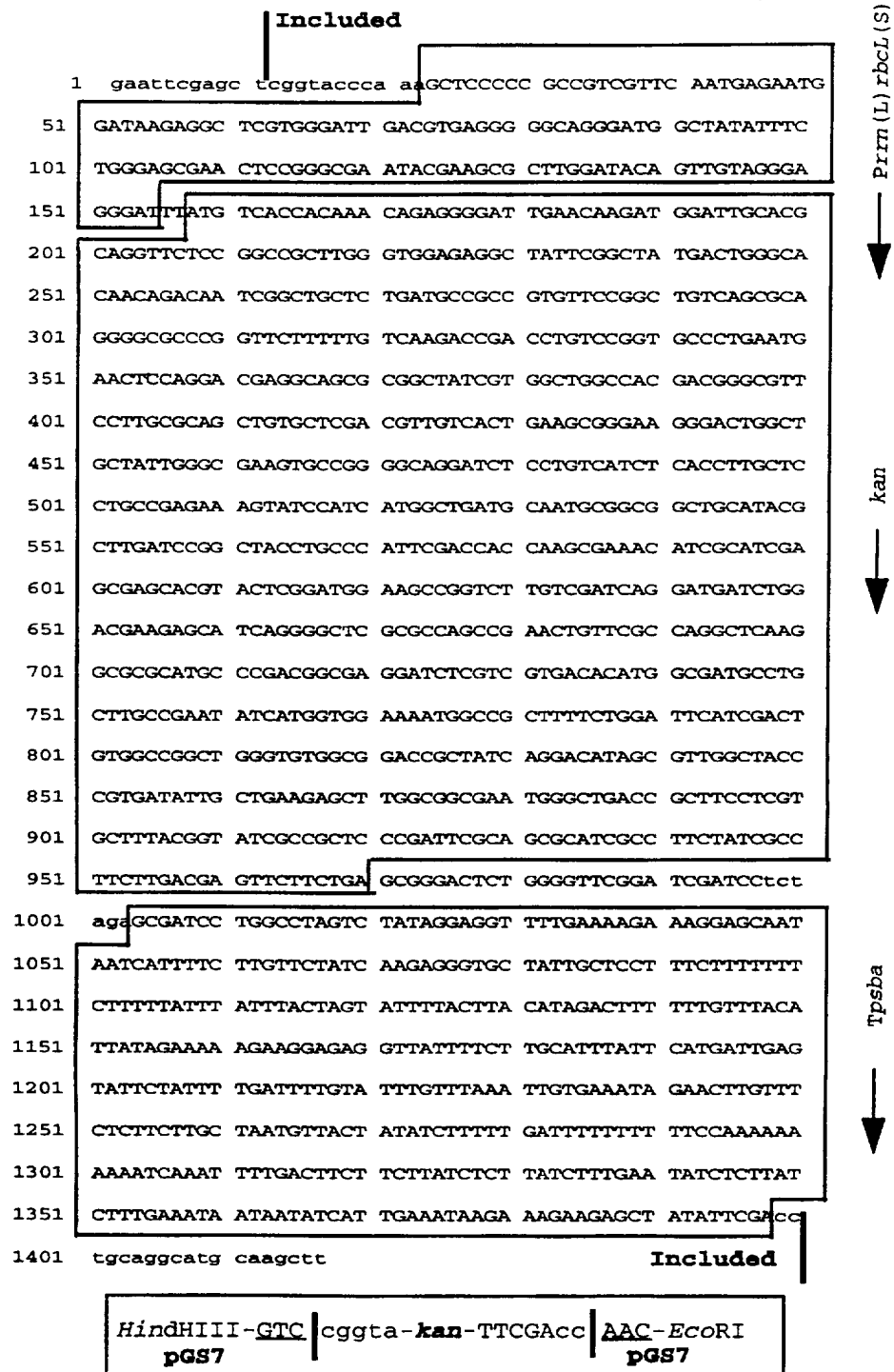


FIGURE 7

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

I. national application No.
PCT/US97/03444

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 15/00, 15/63
US CL : 435/172.1, 320.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.1, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,451,513 A (MALIGA et al.) 19 September 1995, columns 4-5 and 7-12.	1-14
Y, P	US 5,545,817 A (MCBRIDE et al.) 13 August 1996, columns 2-7.	1-14
Y, P	US 5,545,818 A (MCBRIDE et al.) 13 August 1996, columns 2-6.	1-14
Y, P	US (5,576,198) A (MCBRIDE et al.) 19 November 1996, columns 2-11.	1-14
Y	Koop et al. 'Plastid Transformation by Polyethylene Glycol Treatment of Protoplasts and Regeneration of Transplastomic Tobacco.' In: Gene Transfer to Plants. Edited by I. Potrykus et al. New York: Springer-Verlag, 1995, pages 75-82, see entire document.	1-14

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	* & * document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search 27 APRIL 1997	Date of mailing of the international search report 01 JUL 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer THANDA WAI Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

I national application No.
PCT/US97/03444

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, EPO, JPO, BIOSIS, EMBASE, AGRICOLA

search terms: ?plastid?, ?plastom?, transgen?, transplastom?, arabidopsis

INTERNATIONAL SEARCH REPORT

I. .national application No.
PCT/US97/03444

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	STAUB et al. Long Regions of Homologous DNA Are Incorporated into the Tobacco Plastid Genome by Transformation. The Plant Cell. January 1992. Vol. 4, No. 1, pages 39-45, especially pages 39-42.	1-14
Y	STAUB et al. Marker Rescue from the Nicotiana tabacum Plastid Genome Using a Plastid/ Escherichia coli Shuttle Vector. Molecular General Genetics. 1995. Vol. 249, pages 37-42, especially pages 37-40.	1-14
Y	CARRER et al. Kanamycin Resistance as a Selectable Marker for Plastid Transformation in Tobacco. Molecular General Genetics. October 1993. Vol. 241, pages 49-56, especially pages 49-54.	1-14
Y	ZOUBENKO et al. Efficient Targeting of Foreign Genes into the Tobacco Plastid. Nucleic Acids Research. 25 September 1994. Vol. 22, No. 19, pages 3819-3824, especially pages 3819-3822.	1-14
Y	CHAUDHURI et al. Site-Specific Factor Involved in the Editing of the psbL mRNA in Tobacco Plastids. EMBO Journal. 1995. Vol. 14, No. 12, pages 2951-2957, especially pages 2951-2955.	1-14
Y	STAUB et al. Expression of a Chimeric uidA gene Indicates that Polycistronic mRNAs Are Efficiently Translated in Tobacco Plastids. The Plant Journal. 1995. Vol. 7, No. 5, pages 845-848, especially pages 845-847.	1-14